Effect of test duration and feeding on relative sensitivity of genetically distinct clades of *Hyalella azteca*

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Abstract The amphipod *Hyalella azteca* is widely used in ecotoxicology laboratories for the assessment of chemical risks to aquatic environments, and it is a cryptic species complex with a number of genetically distinct strains found in wild populations. While it would be valuable to note differences in contaminant sensitivity among different strains collected from various field sites, those findings would be influenced by acclimation of the populations to local conditions. In addition, potential differences in metabolism or lipid storage among different strains may confound assessment of sensitivity in unfed acute toxicity tests. In the present study, our aim was to assess whether there are genetic differences in contaminant sensitivity among three cryptic provisional species of *H. azteca*. Therefore, we used organisms cultured under the same conditions, assessed their ability to survive for extended periods without food, and conducted fed and unfed acute toxicity tests with two anions (nitrate and chloride) whose toxicities are not expected to be altered by the addition of food. We found that the three genetically distinct clades of *H. azteca* had substantially different responses to starvation, and the presence/absence of food during acute toxicity tests had a strong role in determining the relative sensitivity of the three clades. In fed tests, where starvation was no longer a potential stressor, significant differences in sensitivity were still observed among the three clades. In light of these differences in sensitivity, we suggest that ecotoxicology laboratories consider using a provisional species in toxicity tests that is a regionally appropriate surrogate.

Keywords *Hyalella azteca* · Species complex · Acute toxicity · Nitrate · Chloride

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Introduction

The amphipod *Hyalella azteca* is a useful species for toxicity testing because it is an important component of food webs, it is adaptable to maintenance under laboratory conditions, its size and relatively short life-cycle provide easily measured sub-lethal endpoints for chronic toxicity testing with water or sediment, and it is relatively sensitive to contaminants (Ingersoll et al. 1998; Phipps et al. 1995). However, amphipods that can be morphologically identified as *H. azteca* based on the most recent key (Baldinger 2004) vary widely in size and life history characteristics (Strong 1972; Wellborn 1993), and it is likely that this species name has been assigned too liberally to organisms across North and Central America (Gonzalez and Watling 2002). Indeed, a host of recent studies have documented genetic diversity both within and among wild and laboratory-reared populations of this amphipod, indicating that *H. azteca* is a complex of many cryptic species (Duan et al. 1997; Hogg et al. 1998; Major 2012; McPeek and Wellborn 1998; Thomas et al. 1997; Witt et al. 2006).

Because *H. azteca* is such a widely used toxicity testing organism, it has rightfully been pointed out that caution should be used to identify the particular genetic strain of this species used to generate toxicity data (Duan et al. 1997). Significantly different sensitivities to toxicants have

been observed among different allozyme genotypes within the same population of organisms (Duan et al. 2000a, b, 2001; but see Eisenhauer et al. 1999), which suggests that differences among cryptic provisional species are likely to exist as well. Major (2012) analyzed full cytochrome *c* oxidase subunit I (COI) sequences for 15 research and commercial laboratory populations from the US and Canada, and found that all but one laboratory had organisms with one of two sequences that diverged by only 0.19 % while the remaining laboratory population diverged from the others by ~24 %. While most of the laboratory populations analyzed by Major (2012) could be considered the same species genetically (the US lab clade), she also collected specimens from 22 field populations and identified six distinct provisional species from eastern North Americas based on COI sequences. Furthermore, the provisional species found in most laboratory populations was not commonly found at field sites in eastern North America (four of 22 sites, all in northern Florida). If there are significant differences in contaminant sensitivity among genetically distinct clades of *H. azteca*, researchers may be interested in conducting toxicity tests with a clade that is not only one of the more sensitive ones, but also is one that can be found in the wild in the region of interest.

In the present study, our aim was to assess whether there are genetic (adaptive) differences in contaminant sensitivity among three cryptic provisional species of *H. azteca* identified by Major (2012). It is recommended that acute toxicity studies should be conducted without feeding (ASTM 2002), but if there are distinct differences among provisional species in metabolic rates or fat storage, unfed tests may not accurately portray true differences in contaminant responses. Furthermore, acclimation to local conditions (e.g., water chemistries, ionic compositions, temperature regimes, available food) may mask true genetic differences in stress responses if wild caught organisms are used for these types of assessments (Lam 1999). Therefore, we used organisms cultured under the same conditions, assessed their ability to survive for extended periods without food, and conducted fed and unfed acute toxicity tests with two anions (nitrate and chloride) whose toxicities are not expected to be altered by the addition of food. Both chloride and nitrate are increasingly important contaminants of surface waters. The US Environmental Protection Agency (US EPA) is currently in the process of updating its national water quality criterion for

chloride based on new data (E. Hammer, EPA Region 5, personal communication), and the state of Minnesota is considering adopting water quality standards for protection of aquatic life for nitrate (Monson 2010).

Methods

Clades of *H. azteca* used for testing

Two of our three source populations of *H. azteca* were from laboratory cultures. One was the Illinois Natural History Survey (INHS) culture, which was originally obtained from Aquatic Research Organisms, Inc., in Hampton, NH, USA. This species was genetically identified as the "US Lab clade" by Major (2012), and complete cytochrome oxidase subunit 1 (COI) sequences can be found at Genbank accession nos JX446307 and JX446308. It is also the "species" found in 14 of the 15 laboratory populations investigated in Major (2012). The second of the laboratory populations used in the present study was obtained from Environment Canada's Centre for Inland Waters laboratory in Burlington, Ontario, Canada. This species was genetically identified as the "Burlington clade" by Major (2012), and a complete cytochrome oxidase subunit 1 (COI) sequence can be found at Genbank accession no JX446364. It was the only other "species" found in a laboratory population by Major (2012). The third species was originally collected from Clear Pond, in Kickapoo State Park, Vermilion County, IL. This species was genetically identified as the "Clear Pond clade" by Major (2012), and complete cytochrome oxidase subunit 1 (COI) sequences can be found at Genbank accession nos JX446339 and JX446340. All amphipods were morphologically identified as *H. azteca* based on Baldinger (2004).

Culturing procedures

To eliminate potential differences in sensitivity to contaminants due to acclimation to local conditions or maternal effects, we used organisms from the three clades that had been in culture under the same feeding conditions, temperature, and photoperiod for approximately 2 years (~8–10 generations).

For these experiments, all three clades of *H. azteca* were cultured in a reconstituted water developed at the U.S.

Table 1 Salt concentrations (mg/l) added to deionized water for generation of culture and testing waters for three clades of *H. azteca*

Water name	KHCO$_3$	NaHCO$_3$	MgSO$_4$(an)	CaSO$_4$(an)	CaCl$_2$	NaBr
Duluth 100[a]	10	125	38	40	43	0.05

an anhydrous salt used

[a] Recipe received via personal communication (D. Mount and R. Hockett, US EPA)

Environmental Protection Agency research laboratory in Duluth, MN, (D.R. Mount, personal communication) which will be referred to hereafter as "Duluth 100" water (Table 1). This water recipe was designed with the goal of better mimicking chemistry of "typical" North American freshwaters than other commonly used reconstituted waters. Organisms were cultured at 25 °C, with a 16:8 (L:D) photoperiod. 25 to 30 individuals were held in 1 l beakers with 1,000 ml of water. Nylon screen (www.smallparts.com; 1 mm mesh size) was provided as substrate. At any given time 3–5 beakers were maintained for each clade. Organisms in each beaker were fed daily ∼6 mg of dry, ground and sieved (<250 μm) Tetramin® (TetraWerke, Melle, Germany), and 5.0 mg (dry weight) of mixed diatom solution prepared as described below. Water was changed and screens were cleaned twice weekly; young were counted and collected at that time. Upon collection, young were transferred to a 23 °C environmental chamber for acclimation to test temperature. During this acclimation period, each beaker of organisms was fed ∼3 mg of dry, ground and sieved (<250 μm) Tetramin, and 5.0 mg (dry weight) of mixed diatom solution daily.

Diatoms used to feed amphipods included *Mayamaea* sp., and *Nitzschia* sp. Both diatoms were obtained from Ward's Natural Science (Rochester, NY), sold as *Navicula* sp. and *Synedra* sp., respectively. We had the genus level identities taxonomically confirmed by an expert (S. Decelles) at USEPA-ORD, Cincinnati, OH.

To culture diatoms, we used a modification of the method developed by Weaver et al. (in review). We autoclaved (30 min at 121 °C, liquid cycle) a 4 l flask for each species containing 2 l of filtered (Whatman™ 934-AH) dechlorinated tap water and a 2 inch long, Teflon® coated stir bar. After allowing to cool, sterile technique was used to add 1.3 ml of Kent®—Proculture Professional F/2 Algal culture formula A, 1.3 ml of Kent®—Proculture Professional F/2 Algal culture formula B, 100 mg of sodium metasilicate ($Na_2SiO_3 \cdot 9H_2O$), and 200 ml of fresh diatom stock solution (just removed from stir-plate) for a given species. The flasks were placed on stir-plates with moderate stirring (a minimal vortex was visible) in an environmental chamber set for a 16:8 (L:D) photoperiod and 25 °C. Diatom stocks were allowed to grow for 7 days. Allowing growth for much more than 7 days appeared to cause depletion of nutrients and cell death. After saving 200 ml of stock to seed the next flask, the flask was placed in a refrigerator and allowed to settle overnight. When diatoms had settled, the supernatant was removed, and the remaining solution (∼500 ml) was poured into a 600 ml beaker, and allowed to settle. The supernatant was again removed, and a plastic transfer pipet was used to place concentrated diatoms into a 30 ml polypropylene centrifuge tube (VWR #89012-778). This resulted in a 3–4 ml pellet of diatom cells at the bottom of the tube. Tubes were stored in a refrigerator. Then a calibrated pipet was used to place 4.5 ml from the pellet of each of the single species diatom stocks in centrifuge tubes (see above) into a new, clean, 50 ml polypropylene centrifuge tubes (VWR # 89004-364). Filtered, autoclaved dechlorinated tap water was added until total volume was 30 ml, then the tube was capped and shaken vigorously to mix. The dry weight concentration of the mixed diatoms was measured and recorded on the tube.

Starvation tests

To investigate how lack of food might influence the relative sensitivity of the three clades, we conducted two separate starvation experiments. Both tests were conducted in the same manner. For each clade, we placed one organism into each of 15 replicate 50 ml beakers containing 40 ml of Duluth 100 water and a piece of new nylon screen. Organisms were transferred from culture water to clean water prior to placing in beakers to prevent the transfer of food material from the culture water along with the organism. Organisms were 7–9 days old at the beginning of the test, and all three clades were fed and held in the same manner prior to the test as described above. Test temperature was 23 ± 1 °C and the photoperiod was 16:8 (L:D). Water was not changed over during the test, but because of the lack of food and small size of the organisms relative to the volume of water, we did not expect problems with dissolved oxygen depletion or ammonia accumulation. Organisms were checked daily for mortality and the test was continued until the last organism died (19–21 days).

General acute toxicity testing procedures

We conducted 96 h acute toxicity tests with chloride and nitrate with each of the three clades of *H. azteca* in both fed and un-fed tests. Thus, there were 12 test types (2 contaminants × 3 clades × 2 feeding states), and each test type was conducted three times (to assess variability) for a total of 36 tests. Usually, a test type was conducted with all three clades simultaneously, e.g., unfed, nitrate toxicity tests with all three clades, then fed nitrate toxicity tests with all three clades, etc. Fed and unfed tests were not conducted side by side with the same cohort of individuals, because the Burlington and Clear Pond cultures rarely produced enough individuals to conduct two tests simultaneously. However, because we repeated each test type three times, we feel we were able to observe accurate representations of mean sensitivities and variation thereabout for each test type.

The nitrate and chloride sources for acute toxicity tests were sodium salts (NaNO$_3$ CAS # 7631-99-4; NaCl CAS # 7647-14-5). Static, non-renewal, acute toxicity tests were conducted according to guidelines detailed in American Society for Testing and Materials (ASTM) E729-96 (2002). Treatments were comprised of a 50 % dilution series. Five concentrations were tested in addition to controls. Four replicates were tested per concentration, and five organisms were added to each replicate. Test duration was 96 h with a temperature of 23 ± 1 °C and a 16:8 (L:D) photoperiod. None of the acute tests were aerated. Test chambers were 50 ml glass beakers, and nylon screen was added to each test chamber to provide substrate for these benthic invertebrates. For fed tests, organisms were provided with 0.25 mg (dry weight) mixed diatom suspension on days 0 and 2. Organisms were 7–9 days old at the beginning of the test. Percent survival in each replicate was recorded every 24 h and at the end of the exposure period. The 48 and 96 h median lethal concentrations (LC50s) were calculated using the trimmed Spearman–Karber method (Hamilton et al. 1977). Mean LC50s were compared among test types with analysis of variance (ANOVA) and Student's *t* test was used for post hoc pairwise comparisons. Statistical significance was determined at the α = 0.05 level.

Standard water chemistry parameters were measured at both the beginning and the end of each exposure period, including temperature, pH, conductivity, dissolved oxygen. Alkalinity and hardness were measured at the beginning of the test only. Appropriate meters and methods were used (American Public Health Association 2005). In some cases, water samples from each treatment were collected at the beginning and end of acute tests and submitted to the Water Quality Laboratory in the Department of Agricultural and Biological Engineering, University of Illinois at Urbana-Champaign, for confirmation of nitrate concentrations according to US EPA methods 353.1 (US EPA 1978) and 354.1 (US EPA 1971) or to the IL State Water Survey analytical laboratory for measurement of chloride and sulfate using ion chromatography. Chloride or nitrate was measured in 10 of the 36 tests. For the other 26 tests we compared conductivities at each concentration to those of the same concentrations in measured tests. The overall mean difference between conductivity in measured versus non-measured treatments was 1.57 ± 0.64 %. Supplemental table 1 shows measured conductivity values for all treatments of all tests in relation to measured conductivities for tests in which chloride or nitrate concentrations were measured. To confirm that feeding did not impact toxicity due to differences in dissolved oxygen concentrations, we compared overall mean dissolved oxygen concentrations measured in "out" water samples at the end of fed and unfed tests using Student's *t* test.

Results

General water chemistry

Overall, mean general water chemistry data varied little from test to test. Mean (± standard deviation) temperature, pH, dissolved oxygen, alkalinity, and hardness for all measurements in all tests (both chloride and nitrate tests) were 23.2 ± 0.3 °C, 8.2 ± 0.1, 7.75 ± 0.26 mg/l, 80 ± 1 mg/l as CaCO$_3$, and 82 ± 3 mg/l as CaCO$_3$, respectively. The mean dissolved oxygen concentration for water at the end of fed tests was 7.63 ± 0.18 mg/l, and for unfed tests it was 7.61 ± 0.22 mg/l. These values were not significantly different ($p = 0.8521$), and therefore were assumed not to be a factor in any differences between fed and unfed test results.

Starvation tests

The two starvation experiments had similar results, with the Clear Pond clade being the most sensitive followed by the Burlington and US lab clades (Fig. 1). The Clear Pond clade reached 50 % mortality between days five and six, and the Burlington clade reached the same mark on day 7–8. The US Lab clade appeared to be much more tolerant of starvation, with 50 % mortality observed on day 12 and 14. Unacceptable survival for a toxicity test (<90 %) was
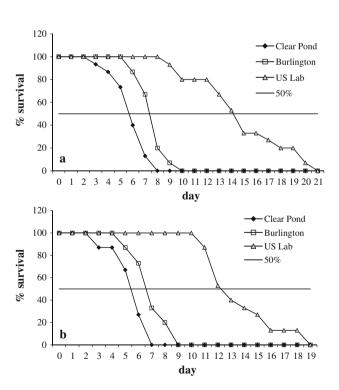

Fig. 1 Percent survival over time of three clades of the *H. azteca* species complex under starvation conditions. Plots **a** and **b** represent two separate experiments

Table 2 Mean[a,b] (± standard deviation) chloride and nitrate 96 h and 48 h LC50s for three genotypes of the *H. azteca* species complex under fed and unfed conditions

Population	Nitrate tests				
	48 h LC50s (mg N–NO$_3^-$/l)			96 h LC50s (mg N–NO$_3^-$/l)	
	Unfed	Fed		Unfed	Fed
US Lab	581 ± 24 a	620 ± 146 a		457 ± 19 B	492 ± 80 B
Burlington	658 ± 18 a	758 ± 57 a		390 ± 80 B	639 ± 62 A
Clear Pond	516 ± 127 a	754 ± 24 a		222 ± 79 C	713 ± 45 A
Population	**Chloride tests**				
	48 h LC50s (mg Cl$^-$/l)			96 h LC50s (mg Cl$^-$/l)	
	Unfed	Fed		Unfed	Fed
US Lab	3,185 ± 190 a	3,103 ± 101 a		2,937 ± 81 A	3,032 ± 85 A
Burlington	2,452 ± 49 b	2,042 ± 70 c		1,530 ± 96 C	1,741 ± 61 C
Clear Pond	2,229 ± 265 bc	2,439 ± 103 b		1,174 ± 126 D	2,117 ± 170 B

Chloride and nitrate data were analyzed separately

[a] Each mean is the average of three tests

[b] Means followed by different lower case (48 h) or capital (96 h) letters are significantly different ($p < 0.05$)

observed in the Clear Pond clade on days 4 and 3 for tests A and B, respectively, while Burlington reached this endpoint on days 6 and 5, respectively. Less than 90 % survival was not observed in the US lab clade until days 10 and 11 of the two tests.

Acute toxicity tests

In all fed and unfed acute toxicity tests with chloride and nitrate, control survival for both the US lab and the Burlington clades was greater than 90 %. For the Clear Pond clade, three unfed tests (with 75, 80, and 85 % survival) and one fed test (with 85 % survival) had less than 90 % control survival at 96 h. Supplemental table 2 shows percent survival at 96 h for all treatments in all tests.

In the nitrate acute toxicity tests, no significant differences among clades or between fed and unfed tests were observed after 48 h (Table 2), with mean LC50s ranging from 516 (unfed Clear Pond) to 758 mg N–NO$_3$/l (fed Burlington). However, at 96 h, significant differences were observed both among clades and within clades in fed versus unfed tests (Table 2). In the unfed tests, the LC50 for the US Lab clade was twofold higher than that of the Clear Pond clade, with the Burlington clade lying between the two. However, the reverse trend was observed in the fed tests with both the Burlington and Clear Pond clades having significantly higher mean LC50s than that of the US Lab. Furthermore, while feeding did not have a significant effect on 96 h LC50 in the US Lab clade, the fed LC50s for both the Burlington and the Clear Pond clade were significantly higher than their corresponding means in unfed tests (Table 2).

For the chloride toxicity tests, significant differences among test types were observed at 48 h. The US Lab clade mean LC50s in unfed and fed tests were significantly higher than tests of either type for the Burlington and Clear Pond clades (Table 2). Interestingly, the Burlington clade mean 48 h chloride LC50 in fed tests was significantly lower than that in unfed tests. The US Lab clade had the highest mean LC50s at 96 h as well. In unfed tests, the mean for the Burlington clade at 96 h was significantly higher than that of the Clear Pond clade, whereas in fed tests, this trend was reversed (Table 2). For both the US Lab and the Burlington clades, feeding did not have a significant effect on 96 h LC50, but for the Clear Pond clade, the mean LC50 in fed tests was nearly double that in unfed tests.

Discussion

In the present study, the three genetically distinct clades of *H. azteca* had substantially different responses to starvation, with the common North American laboratory species (US Lab clade) being far more tolerant than the other two. These three clades had COI sequences that differed from each other by an average of ~23 % (Major 2012). Costa et al. (2009) found that greater than 4.3 % sequence divergence indicated species level differences in the sister genus *Gammarus*; therefore, using that cut-off, Major

([2012](#)) concluded that the three genotypes used in the present study were cryptic but genetically distinct species. In addition to genetic divergence, others have documented differences in life history traits, particularly size class differences, among wild populations of *H. azteca* (Strong [1972](#); Wellborn [1993](#), [1994a](#), [b](#), [1995a](#), [b](#); Wellborn et al. [2005](#)). Based on body size and head capsule length (DS personal observation), the US Lab and Burlington clades would be considered what Wellborn ([1995b](#)) called the large ecomorph of this complex, whereas the Clear Pond clade would be considered the small ecomorph. The smaller size of the Clear Pond organisms might account for their greater sensitivity to starvation, but size does not completely account for the differences because the two larger ecomorphs used in this study had substantially different times to 50 % mortality. This suggests that there may be additional physiological differences, perhaps in food preference, metabolic rate, or lipid storage, among the three clades.

The presence/absence of food during acute toxicity tests with chloride and nitrate had a strong role in determining the relative sensitivity of the three clades. Based on the results of unfed tests, one would conclude that the Clear Pond clade was the most sensitive of the three to both chloride and nitrate, but in fed tests, it was the least sensitive to nitrate and not as sensitive to chloride as the Burlington clade. The fact that the Clear Pond clade had the largest swings in mean LC50s between fed and unfed tests (two- to three-fold) was predicted by the results of the starvation tests. In those tests, this clade reached 50 % mortality shortly after the time when a 96 h toxicity test would end (day 5 or 6), and had less than 90 % survival within 3–4 days. This latter finding was duplicated in the unfed acute tests, where three of the six unfed tests had <90 % control survival after 96 h. One fed test with Clear Pond had 85 % control survival, but the two lowest toxicant concentrations had ≥95 % survival in that test (Table S2). Clearly, lack of food was an additional stressor for the Clear Pond clade.

In contrast to the Clear Pond clade, the results of acute toxicity tests with US Lab clade were not significantly impacted by the presence of food. This is consistent with the fact that in the starvation experiments, this clade did not drop below 90 % survival until more than double the duration of an acute toxicity test (day 10 or 11). The Burlington clade was intermediate between Clear Pond and US Lab in the starvation tests, and food significantly increased its mean LC50 for nitrate but not for chloride.

These results suggest the need to evaluate whether 96 h is the appropriate test duration for acute toxicity tests with the Burlington and Clear Pond clades. If these organisms are dying within 5–8 days under control conditions, the additional stress is likely making them more sensitive to toxicants. We addressed this issue by conducting fed tests as well, but the availability and/or uptake of the contaminants used in this study would not be expected to be altered by food. Trace metals or organic contaminants are likely to interact with food, thereby compromising the results of fed tests with these pollutants. Therefore, because control survival for the Burlington and Clear Pond clades was always ≥90 at 48 h in unfed tests (Table [2](#)), we suggest that 48 h may be the more appropriate test duration for these clades, as is the case for daphnids and midge larvae (ASTM [2002](#)). It is possible that the food combination used for culturing the organisms in these test was not optimal for the Burlington or Clear Pond clades, potentially contributing to their shorter times to starvation. Use of a different food combination in culturing may alter the disparity in responses between fed and unfed tests with those clades; however, our goal was to conduct these tests having exposed all three clades to the same conditions, and that resulted in the observed differences.

In fed tests, where starvation was no longer a potential stressor, significant differences in sensitivity were observed among the three clades. The US Lab clade was significantly more sensitive to nitrate than the other two clades, but its mean chloride LC50 was nearly double that of the Burlington clade and approximately 1.5-fold that of the Clear Pond clade. These findings are not surprising given the studies of Duan et al. ([2000a](#), [b](#)). Those authors used populations that had individuals with six different alleles for three enzymes, but were all the same "strain" or clade at the level discussed in the present study. Upon exposing them to single concentrations of metals, acidic pH, and fluoranthene they observed significantly different probabilities of mortality among the various genotypes. Because they exposed organisms to single concentrations, it is not possible to determine how different LC50s might have been among these genotypes. However, because the three clades in the present study were genetically distinct species rather than allozyme genotypes of the same species, we predict that they would have substantially different mean LC50s for the chemicals studied in the Duan et al. ([2000a](#), [b](#)) studies as well.

Variability in contaminant sensitivity among different genetic strains has been noted in other freshwater invertebrate species as well. For example, Nowak et al. ([2008](#)) had six genetically distinct strains of *Chironomus riparius* in culture for at least two generations, and then compared their sensitivity to cadmium. While Nowak et al. ([2008](#)) did not calculate LC50s, we calculated EC50s for five of their strains using their mortality data in a nonlinear regression. The sixth strain had too little partial mortality to calculate an EC50. Among the five strains, EC50s varied by approximately twofold. Sturmbauer et al. ([1999](#)) documented the presence of different genetic "lineages" within

populations of *Tubifex tubifex* collected from various field sites throughout Eastern Europe. They exposed different populations to cadmium and observed considerable variation in sensitivity. While four of the five populations, had LC50 values that varied by only ~1.5-fold, the sixth population had an LC50 that was ~7-fold lower than the least sensitive population. Numerous studies have been published on the influence of genotype on sensitivity of *Daphnia magna* to toxicants (e.g., Baird et al. 1990, 1991; Barata et al. 2002; Soares et al. 1992). Testing six different genetically distinct clones, Baird et al. (1991) showed that variability in sensitivity among genotypes is dependent on the toxicant tested. For example, there were approximately 32, 12, and sevenfold differences in EC50s among the most and least sensitive clones to cadmium, manganese, and copper, respectively. However, for six other contaminants, EC50s for least and most sensitive clones varied by only 1.4–3.6-fold (average = 2.2).

In the Baird et al. (1991) study, the toxicant most similar in mode of action to those used in the present study was sodium bromide, with an EC50 range of only 1.4-fold between the most and least sensitive genotypes. Similarly, in fed tests from the present study, the 96 h nitrate LC50 for the Clear Pond clade was approximately 1.5-fold higher than that of the US Lab, and the 96 h chloride LC50 for the US Lab clade was 1.7-fold higher than that of the Burlington clade. In unfed tests, the differences between least and most sensitive clades increased to twofold for nitrate and 2.5-fold for chloride. While these differences in sensitivity may not be great enough to cause concern from a risk assessment perspective, they suggest that if more contaminants are tested, one may observe similar degrees of variation to that found for *Daphnia magna* with cadmium, manganese, or copper (Baird et al. 1991).

In assessing sensitivity differences among cryptic species, it is tempting to simply collect organisms from the field and compare their responses in toxicity tests to those of the common laboratory provisional species. However, Lam (1999) suggested that to clearly determine the origin and dynamics of stress tolerance and to accurately predict differential responses of individual populations (or cryptic species), it is instructive to distinguish between genetic differences (adaptation) and differences due to environmental conditions (acclimation). One method Lam (1999) recommended to achieve this distinction was to collect organisms from various populations and to culture them under the same conditions (food, water, temperature, etc.) for one or more (preferably) generations. If differences in sensitivity persist, one can assume that they are genetic and not due to acclimation. That was the approach we took in the present study, having had each of the organisms in culture under the same conditions for approximately 2 years. We feel confident that any previous influence of acclimation to original local conditions was lost in our organisms, and that the responses observed were due to genetic differences among the clades.

In her survey of laboratory and field populations of *H. azteca*, Major (2012) found organisms belonging to the US Laboratory clade at only four of the 22 sites; all of these individuals were collected from springs in northern Florida. No representatives of the US Laboratory clade were identified in any of the remaining locations in Illinois, Michigan, Wisconsin, Vermont or Ontario. Similarly, while a number of other studies have found wild populations with COI sequence fragments that match the clades used in the present study (Dionne et al. 2011; Wellborn and Broughton 2008; Witt et al. 2003, 2006; as determined by Major 2012), none documented the US lab clade east of the Mississippi River. Only one of 15 laboratories sampled by Major had members of the Burlington clade in culture, but individuals belonging to this clade were identified from four field sites in Michigan and one site in Wisconsin. Members of the Clear Pond clade were found in the wild in Illinois, Michigan, Wisconsin and Ontario (Major 2012). More work should be done to truly identify the ranges of these genetic strains in the wild, but it appears that most US and Canadian ecotoxicology laboratories are using a provisional species may be limited in its geographic distribution in eastern North America. Because we observed substantial differences in contaminant sensitivity among the three clades tested in the present study, we suggest that further survey work be done to determine the ranges of these different strains in the wild so ecotoxicology laboratories can consider using a provisional species in toxicity tests that is a regionally appropriate surrogate.

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